Abstract. High molecular weight (HMW) adiponectin (APN) is closely correlated with the development of fatty liver and is modulated by the Akt/forkhead box protein O1 (FOXO1) pathway through disulfide-bond A oxidoreductase-like protein (DsbA-L). The Chinese herb extract, QSHX, is used to treat liver diseases. The present study investigated the effects of QSHX on non-alcoholic fatty liver disease (NAFLD) and its underlying mechanism. A rat model of NAFLD was established by feeding of a high-fat and high-sugar diet for 20 weeks. From week 13, the rats were administered with QSHX, or saline as a control, for 8 weeks. The liver function, blood fat and plasma APN were measured using a radioimmunoassay. The hepatic tissue score was measured following staining for pathology. The expression and activities of Akt, FOXO1, DsbA-L and HMW APN in the adipose tissue and primary adipocytes of the rats were measured using western blot analysis. It was found that QSHX significantly decreased the body weight, liver index, and serum levels of aspartate aminotransferase, alanine aminotransferase and triglyceride; and increased the serum level of APN in the NAFLD rats. Following 8 weeks of treatment with QSHX, the hepatic steatosis in the liver tissue improved and the score of hepatic steatosis was significantly decreased. The results of the western blot analysis indicated that QSHX promoted the expression of DsbA-L and HMW APN, and reduced the expression levels of phosphorylated FOXO1 and FOXO1 in adipose tissue and primary adipocytes. It was concluded that QSHX reduced hepatic steatosis by promoting the expression of HMW APN and DsbA-L, which may have been induced by inhibiting the activation and expression of FOXO1 in adipocytes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is hepatic steatosis caused by the accumulation of excess triglyceride (TG) in the liver. Patients with NAFLD usually exhibit hepatocyte injury and inflammation, which is termed non-alcoholic steato-hepatitis (NASH). NASH is a global chronic liver disease, which is becoming more common, and is closely associated with diabetes and obesity. According to epidemiological findings, at least 1.46 billion adults in the world are obese; in the USA, ~6,000,000 of those with obesity develop NASH and 600,000 suffer NASH-associated hepatic cirrhosis. Increasing data indicate that NAFLD/NASH has high morbidity rates in the Middle East, Far East, Africa, the Caribbean, USA and Latin America. These problems are a significant challenge to human health, however; there is no ideal therapeutic strategy for NAFLD/NASH.

It is reported that adiponectin (APN) is the only cytokine of benefit in NAFLD and its major active form is high molecular weight (HMW) APN. The polymerisation of HMW APN is modulated predominantly by disulfide-bond A oxidoreductase-like protein (DsbA-L). A previous study indicated that the expression of DsbA-L in adipose cells was regulated by the Akt/forkhead box protein O1 (FOXO1) pathway. The extracts of Chinese herbs, termed QSHX, have been used in several diseases and have shown satisfactory effects, particularly for liver diseases in China. The present study investigated the effects of QSHX on liver steatosis in NAFLD rats and its underlying mechanism.

Materials and methods

Chinese herb extract preparation. Dried, sliced crude herbs (6 g Bupleurum falcatum, 6 g Salvia miltiorrhiza, 6 g rhubarb, 6 g lotus leaf, 9 g capillary Artemisia, 15 g rhizome polygoni cuspidate and 15 g gynostemma pentaphyllum) were decocted twice using tap water (40 min each). The decocted solution was filtered through a 150 µm gauze and the filtrate was then concentrated to a mass using a vacuum desiccator at 70°C. The sediment was dried into powder and the powder was dissolved in 25 ml water with 0.9% saline.

NAFLD model establishment and animal treatment. All experiments were approved by the Animal Care and Use...
Committee of Ruikang Hospital Affiliated to Guangxi University of Traditional Chinese Medicine (Nanning, China). A total of 40 adult male Sprague-Dawley (SD) rats of SPF grade (180-200 g) were provided by the Experimental Animal Center of Guangxi Medical University [Certificate of quality no. SCXK (Gui) 2013-0002] and were housed in a restricted access room with controlled temperature (23°C) and a 12-h light/dark cycle. The animals were housed for 3 days prior to experiments, and were randomly divided into a normal control group (n=15), model control group (n=15) and treatment group (n=10). The rats in the normal control group were fed a normal diet, whereas the rats in model control group and treatment group were fed with a high-fat and high-sugar diet (72.8% normal diet + 10% lard + 10% sucrose + 2% cholesterol + 5% yolk powder + 0.2% propylthiouracil) for 20 weeks. The successful establishment of the animal model was confirmed at week 12 by examination of liver pathology in the rats from the normal control group and model control group (n=5 for each group). Water was freely available to the animals during the experiment.

From week 13, the rats in the treatment group were administered intragastrically with dissolved QSHX. The dose administered was 1 ml each day per rat. The rats in the normal control group and model group were administered intragastrically with 1 ml water with 0.9% saline. The administration was performed daily for 8 weeks. The body weights of the rats was measured prior to and following treatment. At the end of the experiment, the wet weight of the liver was measured to calculate the liver index using the following equation: Liver index = liver wet weight/body weight x 100%.

**Serum sample preparation and examination.** Following 8 weeks of treatment, 5 ml blood was collected from the abdominal aorta into a vacuumed anticoagulation tube under anesthesia with 10% chloral hydrate (3.5 ml/kg), which was placed standing for 10 min and centrifuged for 20 min at 1,000 x g at room temperature to collect the serum. The serum was stored in -20°C until the experiments. A TG kit (Donggu Diagnostic Production, Ltd., Zhejiang, China), alanine aminotransferase (ALT)/glutamic-pyruvic transaminase kit (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) and aspartate aminotransferase (AST)/glutamic oxaloacetic transaminase kit (Nanjing Jiangcheng Bioengineering Institute) were used to examine the respective parameters according to the manufacturer's protocol. The serum level of APN was measured using an enzyme-linked immunosorbent assay (ELISA) kit (ab108784, Cambridge, UK) according to the manufacturer's protocol.

**Pathological examination.** The animals were sacrificed by cervical dislocation, following which two sections of hepatic tissue were collected from the same site of the right lobe, fixed in 4% formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). Under an optical microscope, 3 fields (magnification, x200) were randomly selected from each slide to determine the level of hepatic steatosis according to the following scale: 0, no hepatic steatosis; 1, <25% degenerated adipocytes; 2, 26-50% degenerated adipocytes; 3, 51-75% degenerated adipocytes; 4, >75% degenerated adipocytes.

**Protein expression in fat tissues of rats.** The protein from the epididymis fat pad was extracted according to the protocol of MinuteTM Adipose Tissue Fractionation kit (AF-023; Invent Biotechnologies, Inc., Plymouth, MN, USA). The concentration of protein was measured using a bichinonic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Subsequently, samples of 100 g protein were diluted 1:4 with 5X SDS, denatured at 99°C for 5 min and cooled in an ice bath. The samples were then subjected to 8% SDS-PAGE and transferred onto a PVDF membrane, which was incubated with primary antibodies diluted with 5% skim milk overnight at 4°C. The primary antibodies were as follows: GADPH (cat. no. AP0063; 1:5,000; Bioworld Technologies, Inc., St. Louis Park, MN, USA), Akt (cat. no. ab9150; 1:2,000; Abcam, Cambridge, UK), phosphorylated (p)-Akt (cat. no. 2965; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA USA), FOXO1 (cat. no. 2880; 1:10,00; Cell Signaling Technology, Inc.), p-FOXO1 (cat. no. 9461; 1:1,000; Cell Signaling Technology, Inc.), DsbA-L (cat. no. ab92819; 1:2,000; Abcam, Cambridge, UK) and APN (cat. no. bs-0471R; 1:300; Bioss, Beijing, China). The PVDF membrane was then rinsed twice with TBST (5 min each), and incubated with secondary antibodies labelled with HRP (cat. no. BS13278; 1:5,000; BioWorld Technologies, Inc.) for 2 h at room temperature. The bands were developed using the chemiluminescence method and scanned onto film for image analysis.

**Primary cell isolation, culture and differentiation.** In order to determine the effect of QSHX on HMW APN, primary adipocytes of rats were used. Male SD rats in the normal control group were fed for 20 weeks and were sacrificed by cervical dislocation. The intra-abdominal fat pads were excised and the preadipocytes were isolated. Collagenase digestion was performed at 37°C for 1 h and the harvested cells were cultured in DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2 mM penicillin-streptomycin (100 U/ml) and 10% fetal bovine serum (FBS, Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C and 5.0% CO2. The medium was routinely replaced every 2-3 days until the cells reached a confluent state. The preadipocytes were cultured in 12-well plates at 2x10^5 cells/well. The induction of differentiation was performed on day 0 using DMEM supplemented with penicillin-streptomycin (100 U/ml), 10% FBS, 0.5 mM IBMX, 1.0 µM dexamethasone and 5 µg/ml insulin at 37°C in 5.0% CO2. On the subsequent 2 days, the cells were cultured in similar medium but without IBMX or dexamethasone. After 2 days, the cells medium was replaced with DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin. Experiments were performed on days 8-10. Lipid accumulation was examined using Oil-Red-O staining by conventional light microscopy, with stained cells considered to be mature adipocytes for use in the subsequent assays.

**Primary adipocyte assays.** The mature primary adipocytes were obtained using the method described above and cultured in DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin. The QSHX powder was dissolved in DMEM and was passed through a 0.22 µm filter. Following culture of the primary adipocytes for 24 h with DMEM containing QSHX at final concentrations of 4, 2, 1, 0.5, 0.25, 0.125 and 0.625 mg/ml
respectively in 6-well plates (5x10^4 cells/well), the supernatant were collected by centrifugation (500 g for 6 min at 4°C) and APN was measured using ELISA kits (ab108784; Abcam) according to the protocol provided by the manufacturer. Three parallel wells were set up for each concentration. In addition, the adipocytes were cultured with the optimal QSHX concentration, which resulted in the highest level of APN in above ELISA assay for 24 h. The expression levels of HMW APN, Akt, P-Akt, FOXO1, P-FOXO1 and DsbA-L were then examined in the primary adipocytes using western blot analysis. This experiment was performed three times independently.

**Statistical analysis.** The data are expressed as the mean ± standard deviation and were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The comparisons were performed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**QSHX improves the general condition of rats.** During the experiments performed in the present study, the rats in the normal control group were quiet and compliant, with normal hair and normal feeding. The rats in the model control group and treatment group were agitated and aggressive, and exhibited dark hair with hair loss, accompanied with a decrease in feeding from week 4. The changes in the treatment group recovered gradually from week 13. At the end of the experiment, eight rats had survived in each group, respectively.

**QSHX decreases the body weight and liver indices of rats.** No significant difference in body weight was observed between the rats prior to treatment in the treatment group and those in the model control group (280.12±20.13, vs. 273.33±19.35 g, respectively; P=0.503), however, the body weight in the treatment group prior to treatment was significantly increased, compared with that of rats in the normal control group (280.12±20.13, vs. 231.24±18.60 g respectively; P<0.001). Following treatment, the body weight in the treatment group was significantly lower, compared with that in the normal control group (280.12±20.13, vs. 273.33±19.35 g, respectively; P=0.001). The level of APN was also true of liver index (3.21±0.14, vs. 3.56±0.24%, respectively; P=0.006). However, no significant difference was observed between body weight and that of rats in the normal control group (P<0.001; T group vs. M group; “P<0.01, T group vs. M group; **P<0.01, M group vs. CK group. CK, normal control group; M, model control group; T, treatment group.

**QSHX decreases serum ALT, AST, TG, and increases APN in rats with non-alcoholic fatty liver disease.** P<0.05, T group vs. M group; *P<0.05, M group vs. CK group. CK, normal control group; M, model control group; T, treatment group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; APN, adiponectin.

**QSHX reduces hepatic steatosis in NAFLD rats.** Under the microscope, the HE staining in the normal control group (Fig. 3A) showed normal liver lobules with a clear structure, no fat vacuoles and dark colored plasma. In the model control group (Fig. 3B), the liver lobules were not clear in structure, the liver was filled with fat vacuoles of different sizes (fat drops), the collagen around blood vessels was increased and there were few inflammatory cells around the bile conduct. In the treatment group, the liver showed lobules with a clear structure, fat drops of different sizes, increased collagen around blood vessels and few inflammatory cells around the bile conduct (Fig. 3C). Following 8 weeks of treatment, the hepatic steatosis in the treatment group significantly improved and the steatosis score was significantly decreased, compared with the model group (1.79±0.44, vs. 2.57±0.87; P=0.04; Fig. 4).
Effects of QSHX on the Akt/FOXO1 pathway, DsbA-L and HMW APN in adipose tissue. The expression of Akt, p-Akt, FOXO1, p-FOXO1, DsbA-L and HMW APN in adipose tissue are shown in Fig. 5A. The ratio of target protein to GAPDH optical density (OD) was used to analyze the relative expression abundance. The results of the western blot analysis indicated that the expression levels of p-Akt and p-FOXO1 in the treatment group were inhibited by QSHX, the OD value ratios were lower, compared with those in the model control group (0.11±0.03, vs. 0.38±0.08 and 0.24±0.05, vs. 0.54±0.09, respectively; P<0.001). However, the expression levels of p-Akt and p-FOXO1 were significantly higher in the model control group, compared with those in the normal control group (0.38±0.08, vs. 0.28±0.07 and 0.54±0.09, vs. 0.44±0.10, respectively; P<0.005 and P<0.001). The expression levels of DsbA-L and HMW APN in the treatment group were increased by QSHX, with higher OD value ratios, compared with those in the model control group (0.58±0.14, vs. 0.14±0.04 and 0.14±0.02, vs. 0.10±0.03, respectively; P<0.001 and P<0.036). The expression levels of DsbA-L and HMW APN in the model control group were lower, compared with those in the normal control group (0.14±0.04 vs. 0.53±0.10, 0.10±0.03 vs. 0.17±0.05 respectively; P<0.001 and P<0.001). No significant difference was found in the expression of Akt between the normal control group, model control group and treatment group (data not shown), nor was any difference found in the expression of FOXO1 between the normal group and model group (1.20±0.16, vs. 1.22±0.11; P=0.803). However, QSHX significantly inhibited its expression, with OD value ratios of 0.48±0.12 and 1.22±0.11 in the treatment group and model control group, respectively (P<0.001; Fig. 5B).

Effect of QSHX on the secretion of APN in primary adipocyte supernatant. The primary adipocytes were incubated with various concentrations of QSHX for 24 h. The levels of APN were shown in Fig. 6. The lower concentrations of QSHX (0.0625-0.50 mg/ml) increased the secretion of APN, which peaked at 0.5 mg/ml. However, from a concentration 1 mg/ml, the secretion of APN was progressively decreased with increasing concentration of QSHX, and the highest concentration (4 mg/ml) inhibited the secretion of APN. Therefore, the concentration of 0.5 mg/ml was selected for the subsequent experiment.

Discussion

It is advised in clinical practice that patients with NASH or who are at risk from NASH be treated with exercise therapy and diet supplementation with vitamin E or pentoxifylline (6). However, drugs targeting insulin-resistance, including thiazolidinedione and melbene, antioxidants, including vitamin E, anti-fibrotic drugs and pentoxifylline, are all considered experimental treatments as they are not supported by double-blind control experiments. Currently, effective anti-NAFLD/NASH drugs are in the development stage.

APN is one of the adipokines secreted by adipocytes (7). Insulin-resistant experiments in animals and clinical studies have indicated that recombinant APN or whole length of
endogenous APN can decrease the level of blood sugar and accelerate the removal of plasma lipids following consumption of a high-fat diet (8,9). The levels of APN in serum and liver tissues, and the level of APN receptor in the liver tissues of patients with NAFLD are decreased; the decrease in serum APN is more marked in patients with NASH, compared with patients with single fatty liver disease, and are negatively correlated with the level of ALT (10,11). Therefore, APN may be a predictor of the severity of liver disease in patients with NAFLD/NASH (10,11). APN can inhibit NAFLD through increasing the oxidation of fat acid, reducing fat content (12), inhibiting the expression of proinflammatory factors, and the activation, proliferation and migration of hepatic stellate cells (HSCs) (13), and promoting the apoptosis of activated HSCs (14). APN has a similar structure to tumor necrosis factor-α (TNF-α) and shares the same signaling pathway, therefore, APN can antagonize TNF-α to reduce liver inflammation (15). In the present study, a high-fat and high-sugar diet was administered...
APN complex for the synthesis and secretion of APN, and is involved in the formation of HMW APN. It has been found that DsbA-L inhibits the downregulation of APN by endoplasmic reticulum (ER) stress in 3T3-L1 cells, primarily at the protein level (20). Therefore, the present study was performed at the protein level. Wang et al (4) found that the Akt signal is important in modulating the expression of DsbA-L. The treatment of 3T3-L1 cells with the specific Akt inhibitor, AKTI, inhibits the phosphorylation of Akt and downstream FOXO1, but increases the expression of DsbA-L and APN (21).

As a direct substrate of Akt and nuclear transcriptional factor, FOXO1 negatively modulates downstream target genes. Activated Akt (p-Akt) can phosphorylate FOXO1, resulting in the translocation of FOXO1 to the cytoplasm from the nucleus and loss of capability to activate transcription (22). FOXO1 has been confirmed to be involved in the negative modulation of APN (22). The results of the present study indicated that the expression levels of p-Akt and p-FOXO1 were increased in the NAFLD rats, which may result in a decrease of DsbA-L and HMW APN. In the animals treated with QSHX, the expression levels of FOXO1, p-FOXO1 and p-Akt were decreased, whereas those of DsbA-L and HMW APN were increased. In the primary adipocytes of the rats, QSHX also promoted the expression levels of DsbA-L and HMW APN, and inhibited the expression and activation of FOXO1. These results indicated that QSHX inhibits the FOXO1 pathway to increase the expression of HMW APN, which may be one of the mechanisms underlying the effect of QSHX in improving NAFLD.

In conclusion, the present study indicated that QSHX significantly inhibited hepatic steatosis in the NAFLD rats through inhibiting activation of the FOXO1 signal, increasing the expression of DsbA-L and HMW APN. These results provide novel theoretic evidence for QSHX in treating metabolic diseases, including NAFLD, providing a novel therapeutic option for clinicians to treat NAFLD.

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